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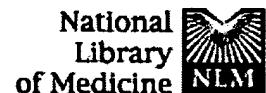
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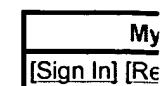
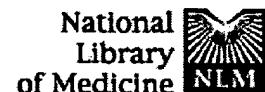


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J Virol. 2003 Mar;77(6):3495-504.
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L20 5 DUP REM L19 (0 DUPLICATES REMOVED)
L21 47 DUP REM L6 (82 DUPLICATES REMOVED)
L22 10 S L21 AND P5

IN Clark, Kelly Reed; Johnson, Philip R., Jr.
SO PCT Int. Appl., 34 pp.
CODEN: PIXXD2
TI Recombinant adeno-associated virus production using recombinant adenovirus or vaccinia virus expressing AAV rep52 and rep40 gene
AB The present invention relates to methods and materials for recombinant adeno-assocd. virus prodn. More particularly, the invention relates to use of recombinant adenovirus encoding adeno-assocd. virus protein in recombinant adeno-assocd. virus prodn. methods. In contrast to previous methods which rely on decreasing the expression of Rep 78 and 68 proteins, the present inventors contemplate that rAAV can be better produced not by intentionally decreasing expression of Rep 78 and 68 proteins, but instead by intentionally overexpressing Rep 52 and Rep 40 proteins and/or providing supplemental Rep 52 and Rep 40 proteins. The invention thus provides an infectious recombinant adeno-assocd. virus-producing cell that contains a rAAV genome, AAV rep-cap proteins and AAV helper functions and that overexpresses AAV Rep 52 and Rep 40 proteins while expressing AAV Rep 78 and Rep 68 proteins at about the level as if controlled by the p5 promoter in native conformation.

IN Chen, Haifeng; Kurtzman, Gary
SO PCT Int. Appl., 84 pp.
CODEN: PIXXD2
TI Production of recombinant AAV using adenovirus vectors comprising AAV rep/cap genes and use thereof
AB This invention relates to novel adenoviruses useful in the prodn. of high titers of recombinant adeno-assocd. virus (rAAV) comprising a foreign DNA insert and methods of making these adenoviruses. The adenovirus comprises the AAV rep gene in which the p5 promoter of rep is replaced by a minimal promoter or by no promoter. The invention also provides methods of producing high levels of rAAV as a substantially homogeneous prepn. and compns. of rAAV.

AU Kube, Dagmar M.; Ponnazhagan, Selvarangan; Srivastava, Arun [Reprint author]
SO Journal of Virology, (1997) Vol. 71, No. 10, pp. 7361-7371.
CODEN: JOVIAM. ISSN: 0022-538X.
TI Encapsidation of adeno-associated virus type 2 Rep proteins in wild-type and recombinant progeny virions: Rep-mediated growth inhibition of primary human cells.
AB The adeno-associated virus type 2 (AAV) arrests the growth of primary human fibroblasts in vitro at high particle-to-cell ratios. To test the role of AAV gene expression in the observed growth inhibition, primary human cells were infected, under identical conditions,

with wild-type (wt) AAV or with recombinant AAV that lacked all viral promoters and coding sequences. Significant, dose-dependent growth inhibition of primary human cells was observed with both wt and recombinant AAV at particle-to-cell ratios equal to or exceeding 10-4. In contrast, neither virus affected the growth of immortalized human cells even at a 10-fold-higher particle-to-cell ratio. AAV-induced growth arrest could be overcome by reculturing cells after treatment with trypsin. Even after reculturing, cells still harbored the proviral AAV genome. Thus, neither integration nor expression of the AAV genome appears to be required for the virus-induced growth-inhibitory effect on primary human cells. The growth-inhibitory effect of AAV was hypothesized to be mediated by virion-associated AAV Rep proteins, since these proteins have been reported to inhibit cellular DNA synthesis. Rep proteins tightly associated with wt as well as recombinant AAV could be detected on Western blots. Coinfection by adenovirus was necessary and sufficient for ample replication of recombinant AAV genomes lacking the rep gene. Although wt AAV-like particles arose during production of the recombinant AAV stocks, their low-titer levels were insufficient to cause the observed growth inhibition. AAV rep gene expression from these contaminating particles was not required for replication of the recombinant AAV genomes, which could be detected even in the absence of de novo Rep protein synthesis. Exposure of recombinant AAV to anti-AAV Rep protein antibodies did not abrogate viral infectivity. These results suggest that biologically active Rep proteins are encapsulated in mature progeny AAV particles. AAV Rep protein-mediated growth inhibition of primary human cells has implications in the use of AAV-based vectors in human gene therapy.

IN Flotte, Terence R.; Carter, Barrie J.; Guggino, William B.; Sollow, Rikki
SO PCT Int. Appl., 50 pp.
CODEN: PIXXD2
TI Generation of high titers of recombinant adeno-associated virus (AAV)
vectors for gene therapy
AB AAV vectors may have utility for gene therapy but heretofore a significant obstacle has been the inability to generate sufficient quantities of such recombinant vectors in amts. that would be clin. useful for human gene therapy application. Stable, helper-free AAV packaging cell lines have been elusive, amainly due to the activities of Rep protein, which down-regulates its own expression and reverses cellular immortalization. This invention provides packaging systems and processes for packaging AAV vectors that effectively circumvent these problems by replacing the AAV p5 promoter with a heterologous promoter and that allow for substantially increased packaging efficiency. Thus, in packaging plasmid pRS5, the AAV p5 promoter is replaced by a heterologous promoter so that expression of the rep gene polypeptide does not neg. autoregulate its own synthesis; pRS5 contains the entire AAV coding sequence within the AAV nucleotide sequence from nucleotide 263 to 4491 which includes the rep coding sequence for Rep 78 and Rep 68 operably linked to the heterologous HIV-LTR promoter and expresses Rep 52 and Rep 40 from the AAV p19 promoter and the AAV capsid proteins from the p40 promoter. The pAAVp5neo construct was used as a vector construct for testing each of the packaging techniques. Human 293-31 cells were grown in Eagle's Modified Essential Medium with 10% fetal calf serum at 37 deg in 5% CO₂. The 293 cell line was used for both packaging of vector preps. and for neo transduction expts. to verify the neo-transducing titers. The pRS5 construct was used to package the pAAVp5neo vector plasmid by cotransfection into adenovirus type 5 (Ad5)-infected 293 cells. An AAV-CFTR vector, pTRF42, contg. the cystic fibrosis transmembrane conductance regulator cDNA expressed from an AAV ITR as the promoter in the absence of a selectable marker was used to generate stable vector producer lines in the 293 cell line by cotransfection with a pSVneo plasmid. This vector was rescuable from the stable cell lines, and the rescued vector was intact and unrearranged. Examples are given on packaging of AAV vectors encoding CFTR, integration of the lacZ gene into lung airway epithelium, expression of the lacZ gene in mice, and integration and expression of the CD44 gene in lung carcinoma cells.

IN Samulski, Richard J.; Xiao, Xiao; Snyder, Richard
SO PCT Int. Appl., 53 pp.
CODEN: PIXXD2

TI Methods for increasing the efficiency of recombinant adeno-associated virus product

AB High-titer stocks of recombinant adeno-assocd. virus (rAAV) are produced in increased yield by regulation of expression of the AAV gene rep and capsid proteins. Low-level expression of the AAV gene rep protein increases the prodn. of AAV viral capsid protein with a resultant packaging efficiency improvement resulting in prodn. of higher titer recombinant viral stocks. Vectors for rAAV direct the expression of AAV gene rep and capsid proteins and are used for the prodn. of novel stable cell lines capable of generating high-titer rAAV vectors. Methods are described for regulating the expression of the AAV rep gene at the transcriptional and post-translational level. Helper plasmids AAV/Ad, CMV/AAV, HIV/AAV, SV/AAV, and ACG-2 were constructed expressing an entire AAV helper plasmid coding sequence including promoter p5, the same sequence with p5 promoter replaced by cytomegalovirus immediate early promoter, by the HIV long terminal repeat promoter, by the SV40 late promoter, and including the promoter p5 but replacement of the initiation codon ATG with the less efficient codon ACG, resp. Human 293 cells were transfected with different ratios of rAAV vector:helper plasmid. There was efficient gene rep expression from heterologous promoters in the absence of adenovirus. Helper plasmids pCMV/AAV and pHIV/AAV generated the lowest rAAV yields, even though they produced the highest Rep levels. Overexpression of gene rep inhibited rAAV DNA replication and capsid gene expression.

IN Colosi, Peter
SO U.S., 19 pp., Cont.-in-part of Ser. No. US 1998-107708, filed on 30 Jun 1998
CODEN: USXXAM

TI Helper functions for adeno-associated virus for high-efficiency generation of wild-type-free virus carrying foreign genes

AB Helper functions for the packaging of adeno-assocd. virus (AAV) that do not allow the generation pseudowild AAV virions are described. The helper functions include the AAV rep and cap genes expressed from the p19 and p40 promoters but lacking a p5 promoter because of deletion of the p5 TATA box. In addn., inverted terminal repeats are deleted from expression constructs for the rep and cap genes. Host cells expressing these genes and the manuf. of transgenic virions are described. A helper plasmid of the invention, pHLPI9, carrying the rep and cap genes and the p19 and p40 promoters gave a yield of AAV that was 200-300% greater than that from prior art helper vectors with no detectable generation of pseudowild type virus.

IN Natsoulis, George; Colosi, Peter; Kurtzman, Gary
SO U.S., 14 pp., Cont.-in-part of U.S. Ser. No. 688,648.
CODEN: USXXAM

TI High-efficiency adeno-associated virus helper functions for improved gene therapy

AB The present invention provides methods and compns. for producing high titer preps. of recombinant AAV ("rAAV") virions. The compns. of the present invention include AAV helper function systems and host cells. The present invention also includes methods of using AAV helper function vectors that effect the prodn. of only small amts. of the long forms of Rep protein, and rAAV virions produced by such methods. Cells transfected with pW1909 comprising rep and cap coding regions and a downstream p5 promoter sandwiched between two Flip Recombination Target (FRT) sites produced very little of the long forms of Rep, as well as more of the short forms of Rep, than other AAV helper function vectors. Introduction of an efficient polyadenylation site between the transcriptional start site and the first codon in the coding sequence of the long forms of Rep protein also decreased expression of the long forms of Rep and

increased expression of the short forms. Prodn. of only small
amts. of the long forms of Rep protein provide for higher titer
rAAV virion prodn.

AU Li J; Samulski R J; Xiao X
SO Journal of virology, (1997 Jul) 71 (7) 5236-43.
Journal code: 0113724. ISSN: 0022-538X.
TI Role for highly regulated rep gene expression in adeno-associated virus
vector production.
AB Recent success achieving long-term in vivo gene transfer without a
significant immune response by using adeno-associated virus (AAV
) vectors (X. Xiao, J. Li, and R. J. Samulski, J. Virol.
70:8098-8108, 1996) has encouraged further development of this vector for
human gene therapy. Currently, studies focus on the generation of
high-titer vectors by using the two-plasmid helper-vector system
in adenovirus (Ad)-infected cells. To examine the effects of
the AAV replication (rep) genes on recombinant AAV (rAAV) vector production, we have constructed a series of
AAV helper plasmids that contain strong heterologous
promoters in place of the endogenous p5 promoter. Although
high-level rep gene expression was achieved,
rAAV DNA failed to replicate in the absence of Ad infection.
Moreover, unregulated overexpression of Rep78/68 led to substantially
lower rAAV yields in the presence of Ad (10(4-5) versus
10(7-8)). In contrast, under similar conditions, reduced Rep78/68
expression resulted in much higher rAAV yields (10(9)).
Molecular characterization showed that overexpression of the rep gene
decreased rAAV DNA replication and severely inhibited capsid
(cap) gene expression. Interestingly, a reduced rep level enhanced cap
gene expression and supported normal rAAV DNA replication.
These studies suggest a critical role for regulated rep gene
expression in rAAV production and have facilitated the
development of a new AAV helper plasmid that increases
vector production eightfold over currently used constructs.

AU Conway J E; Zolotukhin S; Muzyczka N; Hayward G S; Byrne B J
SO Journal of virology, (1997 Nov) 71 (11) 8780-9.
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TI Recombinant adeno-associated virus type 2 replication and packaging is
entirely supported by a herpes simplex virus type 1 amplicon expressing
Rep and Cap.
AB Recombinant adeno-associated virus (AAV) type 2 (rAAV)
vectors have recently been shown to have great utility as gene transfer
agents both in vitro and in vivo. One of the problems associated with the
use of rAAV vectors has been the difficulty of large-scale
vector production. Low-efficiency plasmid transfection of the
rAAV vector and complementing AAV type 2 (AAV
-2) functions (rep and cap) followed by superinfection with adenovirus has
been the standard approach to rAAV production. The objectives
of this study were to demonstrate the ability of a recombinant herpes
simplex virus type 1 (HSV-1) amplicon expressing AAV-2
Rep and Cap to support replication and packaging of rAAV
vectors. HSV-1 amplicon vectors were constructed which contain the
AAV-2 rep and cap genes under control of their native promoters (p5,
p19, and p40). An HSV-1 amplicon vector, HSV-RC/KOS or
HSV-RC/d27, was generated by supplying helper functions with
either wild-type HSV-1 (KOS strain) or the ICP27-deleted mutant of HSV-1,
d27-1, respectively. Replication of the amplicon stocks is not inhibited
by the presence of AAV-2 Rep proteins, which highlights
important differences between HSV-1 and adenovirus replication and the
mechanism of providing helper function for productive
AAV infection. Coinfection of rAAV and HSV-RC/KOS
resulted in the replication and amplification of rAAV genomes.
Similarly, rescue and replication of rAAV genomes occurred when
rAAV vector plasmids were transfected into cells
followed by HSV-RC/KOS infection and when two rAAV proviral
cell lines were infected with HSV-RC/KOS or HSV-RC/d27.
Production of infectious rAAV by rescue from two rAAV
proviral cell lines has also been achieved with HSV-RC/KOS and
HSV-RC/d27. The particle titer of rAAV produced with HSV-RC/d27
is equal to that achieved by supplying rep and cap by transfection
followed by adenovirus superinfection. Importantly, no detectable

wild-type AAV-2 is generated with this approach. These results demonstrate that an HSV-1 amplicon expressing the AAV-2 genes rep and cap along with HSV-1 helper functions supports the replication and packaging of rAAV vectors in a scaleable process.

AU Wang X S; Srivastava A
SO Journal of virology, (1998 Jun) 72 (6) 4811-8.
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TI Rescue and autonomous replication of adeno-associated virus type 2 genomes containing Rep-binding site mutations in the viral p5 promoter.
AB The Rep proteins encoded by the adeno-associated virus type 2 (AAV) play a crucial role in the rescue, replication, and integration of the viral genome. In the absence of a helper virus, little expression of the AAV Rep proteins occurs, and the AAV genome fails to undergo DNA replication. Since previous studies have established that expression of the Rep78 and Rep68 proteins from the viral p5 promoter is controlled by the Rep-binding site (RBS) and the YY1 factor-binding site (YBS), we constructed a number of recombinant AAV plasmids containing mutations and/or deletions of the RBS and the YBS in the p5 promoter. These plasmids were transfected in HeLa or 293 cells and analyzed for the potential to undergo AAV DNA rescue and replication. Our studies revealed that (i) a low-level rescue and autonomous replication of the wild-type AAV genome occurred in 293 but not in HeLa cells; (ii) mutations in the RBS resulted in augmented expression from the p5 promoter, leading to more efficient rescue and/or replication of the AAV genome in 293 but not in HeLa cells; (iii) little rescue and/or replication occurred from plasmids containing mutations in the YBS alone in the absence of coinfection with adenovirus; (iv) expression of the adenovirus E1A gene products was insufficient to mediate rescue and/or replication of the AAV genome in HeLa cells; (v) autonomously replicated AAV genomes in 293 cells were successfully encapsidated in mature progeny virions that were biologically active in secondary infection of HeLa cells in the presence of adenovirus; and (vi) stable transfection of recombinant AAV plasmids containing a gene for resistance to neomycin significantly affected stable integration only in 293 cells, presumably because rescue and autonomous replication of the AAV genome from these plasmids occurred in 293 cells but not in HeLa or KB cells. These data suggest that in the absence of adenovirus, the AAV Rep protein-RBS interaction plays a dominant role in down-regulating viral gene expression from the p5 promoter and that perturbation in this interaction is sufficient to confer autonomous replication competence to AAV in 293 cells.